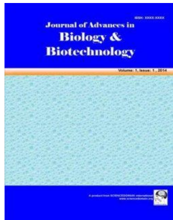

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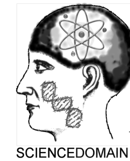

Comparison of Two Biopsy Methods in Bovine Embryos

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Authors' contributions

This work was carried out in collaboration between all authors. Authors YO, TH and TM (the third) designed the study and carried out all experiments. Author YO wrote the first draft the manuscript. Author TM (the fourth) assisted with the writing of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

In this study, the accuracy of sex identification, the viability of biopsied embryos, and the pregnancy rate after biopsied embryo transfer were compared in bovine embryos biopsied by two methods (cutting and pipetting). The cells were more efficiently collected by the pipetting method (2.4±2.0 cells), and the success rate of sex identification was 94.8%. Moreover, the survival, and pregnancy rates of embryo biopsied by pipetting method were 91.7 and 68.0%, respectively, and were numerically higher than those seen in the cutting group (84.4 and 56.5%). From these results, the pipetting method appears to be a simple cell collection method of bovine embryos for sex identification, and assures greater safety of the biopsy process and better embryonic development after biopsy.

Keywords: Bovine; embryo biopsy; embryo transfer; pregnancy; sex identification.

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1. INTRODUCTION

Increasing animal production of a desired gender has important economic implications for the dairy and beef industries. The use of embryo sexing technique can potentially bring greater economic returns for dairy and beef cattle farmers [1]. Developments in embryo micromanipulation techniques have led to the refinement of embryo biopsy procedures which have allowed better bovine embryo sexing [2]. Embryo biopsy can be also used in embryo transfer programs in domestic animals and has potential applications in the identification of genetic markers linked to economic trait loci, and useful for mitochondrial genome analysis, paternity identification and prenatal diagnosis of genetic abnormalities and diseases [3].

In bovine embryos, several biopsy methods have been developed as follows: Excision of trophoblastic cells from the blastocyst using a microsurgical blade [3,4], aspiration of blastomeres from 8-cell stage embryos [5,6], and isolation of degenerating parts of the embryos [7]. In these methods, excision of the trophoblastic cells by a microsurgical blade attached to a micromanipulator (blade cutting method) is the most common [4,8], because the blade cutting method is convenient and allows many embryos to be processed within a short time. This method is also very popular for sampling bovine embryos to identify sex in Japan. However, embryonic biopsy using a micromanipulator is necessary to master high levels of manipulation skills [9]. It is also a problem because micromanipulators are expensive. Moreover, it is noted that the biopsy technique is directly related to embryo viability and conception rate after embryo transfer [4,9,10]. Therefore, a new biopsy method without harmful effects to embryo viability and conception rate should be developed.

In this study, we attempted to perform biopsy from bovine embryos by pipetting and then examined the accuracy of sex identification, the viability of biopsied embryos, and the pregnancy rate after biopsied embryo transfer and compared the results with embryos treated with the cutting method.

2. MATERIALS AND METHODS

2.1 *In vitro* Embryo Production

The ovaries of Japanese black or Holstein cows were used in this experiment. A part of the ovaries for oocyte collection were derived from a slaughterhouse, but the majority of ovaries were from living cows reared in the Hiroshima Prefecture Livestock Technology Research Center. The cows were handled in accordance with the regulations set by the Hiroshima Prefecture Livestock Technology Research Center for animal experiments.

Ovaries from the slaughterhouse were brought to the laboratory in physiological saline supplemented with 0.1 mg/ml kanamycin (Kanamycin sulfate; Meiji Seika, Tokyo, Japan). The oocytes with follicular fluids were aspirated from the ovarian follicles (2-10 mm in diameter) by a 5 ml syringe fitted with an 21G 5/8 gauge needle. Follicular fluids with oocytes were collected in 10 ml conical tubes with m-PBS supplemented with 10% FCS (Hyclone; Funakoshi K.K., Tokyo, Japan) which were warmed at 38°C up to the time of oocyte search.

Oocyte collection from living cows was performed once every two weeks by ultrasound-guided transvaginal follicular puncture technique (OPU). Prior to follicular aspiration, the donor cows received 4 ml of epidural anaesthetics (Adosan; Riken K.K., Tokyo, Japan) to prevent abdominal straining and to relax the rectum, which was necessary for palpation of the ovaries for a long time. In follicular aspiration, we used a real-time ultrasound scanner (SSD-1200 type; Aloka Co. Ltd., Tokyo, Japan) and a 7.5 MHz convex array transducer (UHT-9106 type; Aloka) attached with a 17-gauge disposable stainless steel needle (COVA Needle, Misawa Medical, Tokyo, Japan). Follicles over 2 mm in diameter were aspirated by an aspirator (K-MAR-5115 type; Cook Medical Technology, Australia) equipped with a needle. The aspiration rate and vacuum pressure were 20 ml/min and 115 mmHg, respectively. Follicular fluids were collected in 50 ml conical tubes (Sumilon; Sumitomo Bakelite Co. Ltd., Tokyo, Japan) maintained at 35°C by a warmer (Model FV5; FHK, Tokyo, Japan) until oocyte collection. Lactate Ringer solution (Haruzen V Zenoaq, Fukushima, Japan) supplemented with 0.3% FCS (Funakoshi), with 10 units/ml heparin (NeotubeNipro; Osaka, Japan) and 0.1 mg/ml kanamycin (Meiji Seika Pharma Co. Ltd., Japan) was used for collection medium. Cumulus-oocyte complexes (COCs) were isolated from the follicular aspirates.

Quality was graded according to classical morphologic criteria as described [11]. Briefly, Grade A COCs had compact, multilayered

cumulus cells with homogeneously organized ooplasm; Grade B COCs had compact cumulus cells with homogeneously organized ooplasm; Grade C COCs had less compact cumulus cells with irregular ooplasm containing dark clusters in the ooplasm; and Grade D COCs were without cumulus cell or had over-expanded cumulus cells and a jelly-like matrix. All except Grade D COCs were used for *in vitro* maturation (IVM). The COCs were washed 3 times with M2 solution (M-5910; Sigma Aldrich Japan, Tokyo, Japan) supplemented by 20% FCS (Funakoshi). IVM was performed in 25 mM HEPES-buffered TCM199 (12340-030; Gibco, Grand Island, NY, USA) supplemented with 20% FCS (Funakoshi), 50 ng/ml EGF (E-1264; Sigma), 0.12 mg/ml FSH (Kyoritsu Seiyaku Corporation, Tokyo, Japan). The COCs were incubated for 20-22 h at 38.5℃ in humidified atmosphere of 5% CO_2 in air. These procedures were basically performed according to the method described by Horiuchi et al. [12].

In vitro fertilization (IVF) was performed on matured oocytes. Oocytes were coincubated with spermatozoa at a concentration of 12 x 10^6 for 6 h, and then the cumulus cells were removed. All zygotes were transferred into a culture medium (m-SOF) supplemented with 3 mg/ml BSA (Sigma), 0.25 mg/ml linoleic acid albumin (L-8384; Sigma) in 100 µl droplets covered with mineral oil, and incubated for 72 h at 38.5°C in humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2. Then, they were co-cultured on a monolayer of Vero cells in a 4-well culture dish (Nunc, Roskilde, Denmark) with m-SOF culture medium supplemented with 10% FCS (Funakoshi) and 0.25 mg/ml linoleic acid albumin (Sigma) at 38.5°C in humidified atmosphere of 5% CO_2 in air.

2.2 Cell Sampling by Embryo Biopsy

Two methods (blade cutting and pipetting) for cell sampling from embryos were adopted in this experiment. In the blade cutting method, blastocyst-stage embryos cultured *in vitro* for 7 days after fertilization were used. M2 medium (50µl, Gibco) supplemented with 20% FCS (Funakoshi) and 0.1 M sucrose (WakoPure Chemical Industries Ltd., Tokyo, Japan) was put in a petri dish (30 mm in diameter), and covered with mineral oil. The dish was set on an inverted microscope (Diaphoto 300; Nikon, Tokyo, Japan) equipped with a micromanipulator (M type; Leitz, Germany) fitted with a micro blade (Micro Feather Blade K-730, Feather Co. Ltd., Tokyo, Japan). Approximately 1/3 of the blastocyst (trophoblastic cells) was removed by cutting with the blade. The biopsied embryos were transferred into another M2 medium with 20% FCS (Funakoshi), and then washed 6 times. The embryos were co-cultured on a monolayer of Vero cells in a 4-well culture dish (Nunc) with m-SOF culture medium for 2-3 h at 38.5ºC in humidified atmosphere of 5% CO_2 in air. Blastocoel formation was morphologically ascertained. More than 200 embryos at blastocyst stage were cut by a blade.

In the pipetting method, morula-stage embryos cultured *in vitro* for 5 days after fertilization were used. The embryos were treated with 0.25% pronase (Actinase E; Kaken Seiyaku Co. Ltd., Tokyo, Japan) in m-PBS for 60 sec to dissolve the zona pellucida. The zona free embryos were transferred into M2 medium supplemented with 20% FCS (Funakoshi) to stop enzyme reaction.The embryos were placed into a droplet of m-PBS supplemented with 0.125% trypsin (27250-042; Gibco) and 0.02% EDTA4Na (Sigma), and then were gently pipetted several times by a sharpened capillary glass (057910; Drummond, Broomal, PA, USA) of about 110 µm in diameter.When several outer blastomeres were detached from the embryo, the embryos were transferred in a M2 medium supplemented with 20% FCS (Funakoshi). More than 500 embryos at morula stage were biopsied by pipetting method. The biopsied embryos were co-cultured on a monolayer of Vero cells in a 4-well culture dish (Nunc) containing m-SOF culture medium for 48 h at 38.5°C in humidified atmosphere of 5% CO_2 in air.

2.3 Sex Identification

The sample cells (trophoblastic cells or blastomeres) were washed 6 times with a M2 medium supplemented with 20% FCS (Funakoshi), followed by washing 6 times with a PBS(-) solution supplemented with 1 mg/ml polyvinyl alcohol (30,000-70,000 M.W.; Wako). Finally, the cells were washed once with sterile water. Sex identification of the samples was conducted by loop-mediated isothermal amplification (LAMP) method using a bovine sexing kit (Eiken Chemical Co. Ltd., Tochigi, Japan) according to the manufacturer's instructions. The procedure was described by Agung et al. [13] in detail.

The numbers of biopsied sample cells were counted by the fluorescence staining method as

described previously by Pursel et al. [14].The sample cells were stained in 10 ng/ml Hocehst 33342 solution (Dojindo Molecular Technologies Inc., Kumamoto, Japan). Fixed and stained whole blastocysts were mounted and assessed for cell number using a ultraviolet fluorescent microscopy. The blastocysts were then mounted on a glass microscope slide in a fluorescence staining solution (fixative solution of 100% ethanol with 10 ng/ml Hoechst 33342), gently flattened with coverslip and visualized for cell counting.

2.4 Embryo Transfer and Pregnancy Diagnosis

The embryos (n=67) of determined sex were transferred into the uterus horn of synchronized recipient heiferson day 7 by standard non-surgical embryo transfer procedures. Numbers of embryos transferred cows are 19, 23 and 25 in control, cutting and pipetting groups, respectively. Pregnancy diagnosis was performed ultrasonographically on day 23 after embryo transfer. Pregnancy was judged by the presence of uterine cavity and/or fetus heart beating.

2.5 Statistical Analysis

The number of biopsied cells and embryonic cell numbers after biopsy were statistically analyzed by Welch's t-test and Post-hoc Scheffe's F-test, respectively. The success rate of sex identification and survival rate of biopsied embryos were analyzed using chi-square tests. The pregnancy rate was compared by using Kruskal-Wallis test. Probability of $P<0.05$ was considered to be statistically significant.

3. RESULTS

Embryos biopsied by pipetting and cutting methods were shown in Fig. 1.

Embryo biopsy by pipetting method can be accomplished in less than 3 min per embryo, while it took about 15 min for processing of cutting including blade changing.

As shown in Table 1, the number of biopsied cells by two methods (cutting and pipetting) were 24.8±14.0 and 2.4±2.0, respectively. A significant difference ($P<.05$) was observed between them.

Table 1. Number of biopsied cells from embryos

Treatment	No. of treated embryos	No. of biopsied cells (mean ± S.D.)
Cutting	121	24.8 ± 14.0^{a}
Pipetting	241	2.4 ± 2.0^{b}

[a,b] Values with different superscripts are significantly different (P<.05)

The success rates of sex identification by LAMP method were 96.3% and 94.8%, respectively (Table 2). No significant difference was observed between them.

The proportion of male and female in the collected cells is shown in Table 3. The sex ratios in both treatments were almost 50%.

Embryonic cell numbers in untreated (control) and treated (cutting and pipetting) groups are presented in Table 4. The numbers in treated groups were significantly ($P<.05$) lower than the control. Additionally, the number in the cutting group was significantly ($P<.05$) lower than that in the pipetting group.

As shown in Table 5, the survival rates in the cutting and pipetting groups were 84.4% and 91.7%, respectively. No significant difference was observed between them.

The effect of untreated and treated (cutting and pipetting) embryos following embryo transfer on pregnancy rate is shown in Table 6. The rates of untreated and treated (cutting and pipetting) groups were 68.4%, 56.5% and 68.0%, respectively. No significant difference was obtained among them; however, the rate in the cutting group was numerically lower than those in other groups.

Table 2. Success rate of sex identification

Treatment	No. of treated embryos	Determined	Undetermined	Success rate (%)
Cutting	190	183	7	96.3
Pipetting	520	493	27	94.8

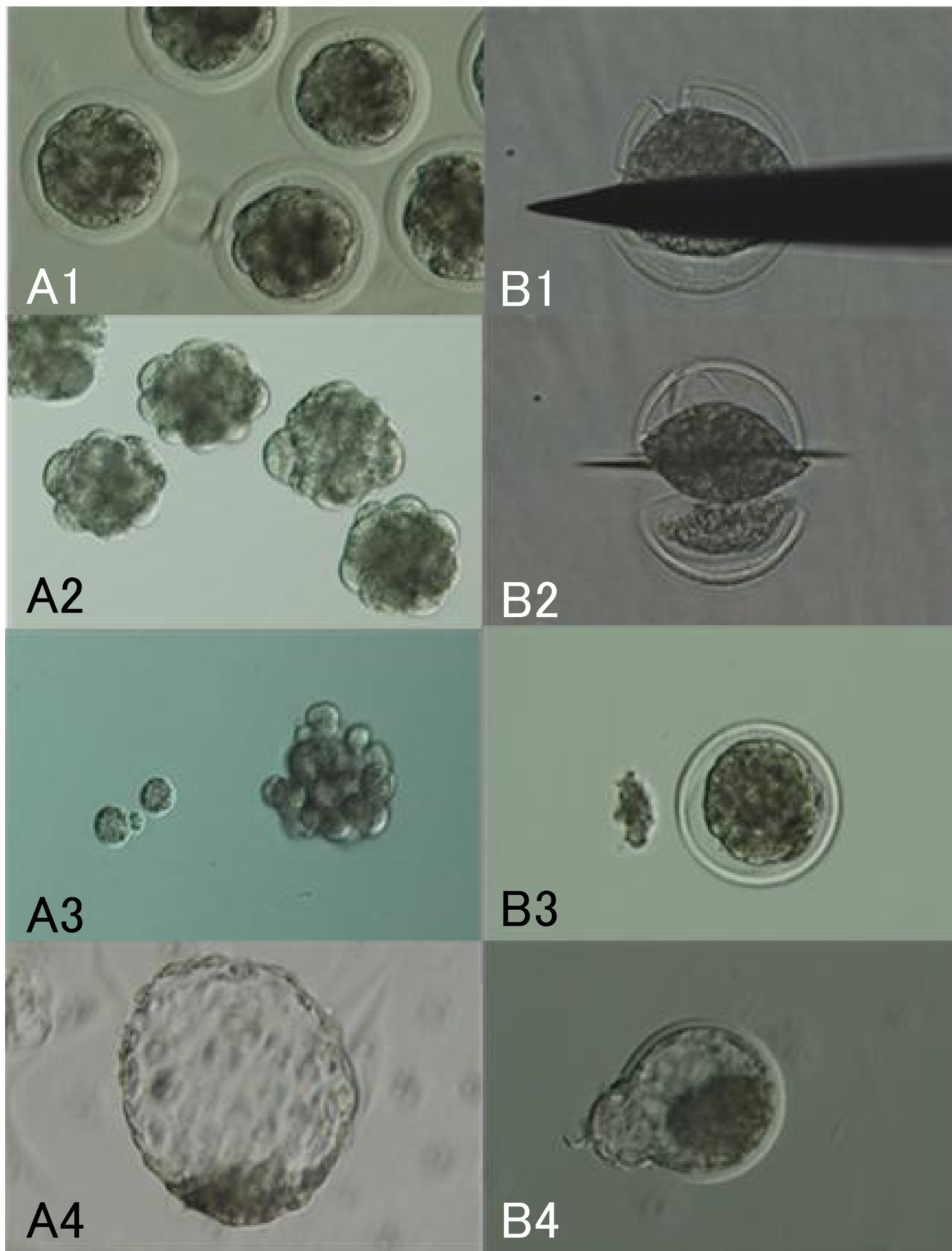

Fig. 1. Embryos biopsied by pipetting and cutting methods

A1: Morula-stage embryos for pipetting treatment; A2: Embryos treated with pronase; A3: An embryo after pipetting treatment and detached outer blastomeres; A4: A blastocyst-stage embryo cultured for 48 h after pipetting treatment; B1: A blastocyst-stage embryo and a cutting blade; B2, B3: An embryo cut by a blade; B4: A blastocyst-stage embryo cultured for 2 h after cutting

Table 3. Proportion of male and female embryos

Treatment	No. of treated embryos	Male (%)	Female (%)
Cutting	183	88 (48.1)	95 (51.9)
Pipetting	493	242 (49.1)	251 (50.9)

Table 4. Embryonic cell numbers after biopsy

Treatment	No. of treated embryos	Cell numbers (mean ± S.D.)
Untreated (Control)	103	117.5 ± 29.9[a]
Cutting	103	77.3 ± 24.7[b]
Pipetting	100	96.2 ± 26.6[c]

[a,b,c] *Values with different superscripts are significantly different (P<.05)*

Table 5. Survival rate of embryos after biopsy

Treatment	No. of biopsied embryos	No. of surviving embryos*	Survival rate (%)
Cutting	122	103	84.4
Pipetting	109	100	91.7

**The embryo developing into blastocyst with blastocoel after culturing for 2-3 h was defined as survival*

Table 6. Effect of different biopsy treatments on pregnancy rate following embryo transfer

Treatment	No. of embryo transferred cows	No. of pregnant cows	Pregnacy rate (%)
Untreated (Control)	19	13	68.4
Cutting	23	13	56.5
Pipetting	25	17	68.0

4. DISCUSSION

The cutting of embryonic trophectoderm cells using a blade (cutting method) has been widely used for the biopsy of bovine embryos [4,7,8]; however, a few problems were encountered as follows: decreased developmental potential of biopsied embryos and complexity of washing blade [9,10,15]. In this study, the accuracy of sex identification, the viability of biopsied embryos, and the pregnancy rate after biopsied embryo transfer were compared when bovine embryos biopsied by two methods (cutting and pipetting). Embryo biopsy by pipetting method could be accomplished in less than 3 min per embryo. Cells were more efficiently collected by the pipetting method (2.4±2.0 cells), and the success rate of sex identification was 94.8%. Moreover, the survival and pregnancy rates of biopsied embryos using the pipetting method were 91.7 and 68.0%, respectively. These rates were numerically higher than those in the cutting group (84.4 and 56.5%). This pregnancy rate was almost similar to other reports employing embryo transfer using biopsied embryos [4,10,16]. From these results, it was considered that the pipetting method might be more efficient than the cutting method.

Chrenek et al. [17] demonstrated a procedure for multiple genotype analysis (determination of sex and of three genetic markers) from a single cell derived from bovine pre-implantation embryo. Other studies [18,19,20] have shown that the biopsy of single blastomere was enough for performing sexing and other genotype analysis. As more than two cells were certainly collected by the pipetting method in this study, the present method would be useful for genomic diagnosis in bovine pre-implantation embryos.

The present study has demonstrated that the pregnancy rate of the cutting group was numerically lower than that of the pipetting group. This seems to be caused by inflicting bigger damage to embryonic cells by cutting during biopsy. In fact, the number of cells collected from the embryos treated with cutting (24.8±14.0 cells) was significantly higher than that of treated with pipetting (2.4±2.0), and embryonic cell numbers after biopsy in the cutting group (77.3±24.7 cells) was significantly lower than that in the pipetting group (96.2±26.6). These results agree with the report by Vajta et al. [5] who demonstrated the overall efficiency of *in vitro* production-biopsy-cryopreservation in bovine embryos.

Concerning the accuracy of sex identification, the values reported by Machaty et al. [21], Thibier et al. [4], Lopes et al. [22], and Hirayama et al. [8] were 83, 94.7, 96, and 97.3%, respectively. The accuracy of sex identification in the present study were 96.3% and 94.8%, making these values closely similar to previous reports.

5. CONCLUSION

The present study showed a simple cell collection (pipetting) method from bovine embryos for sex identification. This method is rapid, and does not need an expensive

micromanipulator. This method can assure the safety of the biopsy process and results to better embryonic development after biopsy in bovine embryos.

ETHICAL APPROVAL

The authors declare that this work was not against public interest.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Geshi M. Current status and the future of bovine embryo transfer using sex determined embryos. J Mamm Ova Res. 2012;29:124-7.
2. Lopes RE, Forell F, Oliveira AT, Rodrigese JL. Splitting and biopsy for embryo sexing under field conditions. Theriogenology. 2001;56:1383-92.
3. Leoni G, Ledda S, Bogliolo L, Naitana S. Novel approach to cell sampling from preimplantation bovine embryos and its potential use in embryonic genome analysis. J Reprod Fert. 2000;119:309-14.
4. Thibier M, Nibart M. The sexing of bovine embryos in the field. Theriogenology. 1995;43:71-80.
5. Vajta G, Holm P, Greve T, Callesen H. Comparsion of two methods to produce *in vitro* fertilized, biopsied and vitrified bovine embryo. Theriogenology. 1997;47:501-9.
6. Shirazi A, Borjian S, Nazari H, Ahmadi E, Heidari B, Bahiraee A. Effects of timing on cell biopsy from pre-compacted morula stage bovine embryos on subsequent embryonic development. J Reprod Infertil. 2010;11:25-32.
7. Lopatarova M, Cech S, Krontorad P, Holy L, Hlavicova J, Dolezel R. Lower quality bovine embryos may be successfully used for sex determination. Veterinarni Medicina. 2007;52:540-6.
8. Hirayama H, Kageyama S, Moriyasu S, Hirano T, Sugimoto Y, Kobayashi N, et al. Genetic diagnosis of claudin-16 deficiency and sex determination in bovine preimplantation embryos. J Reprd Dev. 2004;50:613-8.
9. Mapletoft RJ, Hasler JF. Assisted reproductive technologies in cattle: A review. Rev Sci Tech Int. 2005;24:393-403.
10. Schmidt M, Avery B, Smith SD, Purwantrara B, Greve T. The freezability of biopsied bovine embryos. Theriogenology. 1992;38:615-21.
11. Loos DF, Viliet C, Maurik P, Kruip AM. Morphology of immature bovine oocytes. Gamete Res. 1989;24:197-204.
12. Horiuchi T, Emuta C, Yamauch Y, Oikawa T, Numabe T, Yanagimachi R. Birth of normal calves after intracytoplasmic sperm injection of bovine oocytes: A methodological approach. Theriogenology. 2002;57:1013-24.
13. Agung B, Otoi T, Wongsrikeao P, Taniguchi M, Shimizu R, Watari H, et al. Effect of maturation culture period of oocytes on the sex ratio of in vitro fertilized bovine embryos. J Reprod Dev. 2006;52:123-7.
14. Pursel VG, Wall RJ, Rexroad CE Jr, Hammer RE, Brinster RL. A rapid whole-mount staining procedure for nuclei of mammalian embryos. Theriogenology. 1985;24:687-91.
15. Skrzyszyswska M, Smorag Z. Effect of splitting on cell losses and the quality of bisected embryos. Theriogenology. 1987;27:276.
16. Lopatarova M, Cech S, Krontorad P, Holy L, Lalova H, Dolezel R. Conception rate after sex determination and cryopreservation of D7 bovine embryos. Veterinarni Medicina. 2010;55:10-8.
17. Chrenek P, Boulanger L, Heyman Y, Uhrin P, Laurincik J, Bulla J. Sexing and multiple genotype analysis from a single cell of bovine embryo. Theriogenology. 2001;55:1071-81.
18. Park JHJ, Lee H, Choi KM, Joung SY, Kim JY, Chung GM. Rapid sexing of preimplantation bovine embryo using consecutive and multiplex polymerase chain reaction (PCR) with biopsied single blastomere. Theriogenology. 2001;55:1843-53.
19. Carneiro MCA, Takeuchi PL, Araujo A, Lobo RB, Elias FP, Vila RA, Miranda-Furtado CL, Ramos ES. Sexing single bovine blastomeres using TSPY gene amplification. Genet Mol Res. 2011;10:3937-41.
20. Zoheir KM, Allam AA. A rapid method for sexing the bovine embryo. Anim Reprod Sci. 2010;119:92-6.

21. Machaty Z, Paldi A, Csaki T, Varga Z, Kiss I, Barandi Z, et al. Biopsy and sex determination by PCR of IVF bovine embryos. J Reprod Fert. 1993;98:467-70.
22. Lopes da Costa L, Chagas e Silva J, Diniz P, Cidado R. Preliminary report on sexing bovine pre-implantation embryos under the conditions of Portugal. RPCV. 2002;97:95-8.

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